FINAL REPORT

BDF PROJECT #98000360

"Vaccine Delivery For Oral Immunization of Neonatal Calves"

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SUMMARY

The objective of this project is to develop an oral vaccine delivery system for the immunization of newborn calves. An effective oral vaccine delivery system must be able to protect the vaccine from degradation during passage through the stomach and ensure efficient targeting of the vaccine to the immune system (mucosa-associated lymphoid tissue) in the small intestine. The research in this project involved designing a system to produce solid particles (alginate microspheres) for vaccine delivery and then using these alginate microspheres to formulate vaccine antigens. The efficacy of this vaccine delivery system was then evaluated by delivering oral vaccines to young calves and evaluating specific immune responses to the vaccine antigens.

There were three specific Aims in the project proposal. These Aims were: 1) establish a microsphere production system; 2) target microspheres to the gut-associated lymphoid tissue; and 3) test the efficacy of microspheres for oral vaccine delivery. In the previous reporting periods we completed Aims 1 and 2 and in the present reporting period we addressed Aim 3. In previous reporting periods we established a production unit and standard protocol to produce alginate microspheres. The uptake of alginate microspheres by gut-associated lymphoid tissue (Peyer's patches) was then established and we then confirmed that uptake of alginate microspheres resulted in the induction of a mucosal immune response in the small intestine.

In the present reporting period we addressed Aim 3 in which we proposed to determine if alginate microspheres provided an effective oral vaccine delivery system for a bovine adenovirus 3 (BAV3) vaccine vector. Experiments performed with intestinal "loops" in young calves revealed that the BAV3 vaccine vector did not induce a vaccine-specific immune response. Therefore, we selected an alternative, commercial rotavirus vaccine antigen (P1P5). Assays were established to detect P1P5-specific immune responses and P1P5 protein was then expressed using an E. coli bacterial expression system. The E. coli expression system induced P1P5-specific immune responses in both the intestinal "loop" model and following oral immunization of young calves. Therefore, this system appears to have significant potential as a commercial oral vaccine delivery system.

TECHNICAL REPORT

Background

Approximately 75% of all mortality, for cattle less than one-year old, occurs during the first four weeks after birth. The high prevalence of disease in newborn calves has been associated with: **1)** failure of passive transfer of maternal immunity; **2)** management procedures that favour disease transmission; and **3)** an immature immune system. Cost-benefit analyses indicate that immunization would be an economic way of controlling disease in newborn calves. However, vaccines are thought to be ineffective in newborn calves because of interference by maternal antibodies and a less reactive, immature immune system in newborn calves. These concepts have been challenged by our recent results, demonstrating that oral vaccination of newborn lambs induced a strong immune response. In fact, the immune system in the gut of most domestic animals is fully developed prior to birth. Furthermore, recent investigations completed at VIDO demonstrated that maternal antibody in colostrum did not inhibit the capacity of a human adenovirus vaccine vector to induce active immunity in the intestine of newborn lambs. Thus, it should be possible to provide much better disease protection by combining the protection of maternal antibody with a vaccine induced immune response.

It has long been recognised that over 95% of all disease causing organisms enter the body through the mucosal surfaces of the gut (oral transmission), lungs (aerosol transmission), or reproductive tract (venereal transmission). Furthermore, it is apparent that intramuscular/subcutaneous vaccines induce strong immune responses in the blood but very poor immune responses at the mucosal surfaces where disease organisms enter the body. Recent investigations have clearly determined that to induce a strong protective response at mucosal surfaces it is necessary to delivery vaccine antigens directly to the mucosal surfaces. Oral immunization is about ten-fold more efficient in generating specific intestinal immunity than intramuscular injection and mucosal immunity was found to be the most important correlate of protection against rotavirus infection in the intestine. It is difficult to deliver vaccines to mucosal surfaces and this has limited the development of this type of vaccine for domestic animals. An intranasal IBR (bovine

herpesvirus-1) vaccine is available for cattle. This modified-live virus vaccine is very effective but has not received widespread producer acceptance due to the difficult intranasal delivery. An oral vaccine should be much easier to administer to newborn calves and could fit well with management procedures completed at this time (ear tagging, dehorning, and castration).

Bovine adenovirus type 3 (BAd3) was isolated from the conjunctiva of the eye of a healthy cow and infection of calves with BAd3 resulted in viral replication with only mild or no clinical symptoms. Adenoviruses have been shown to be effective for use as vaccine vectors in a variety of animal models. These studies demonstrated that adenovirus vaccine vectors are an effective means of inducing mucosal immunity and in these studies the adenovirus vectors were delivered either intranasally or orally. The BAd3 virus has been fully characterized at VIDO and the virus is easily produced in tissue culture. This is important for the production of a vaccine virus.

Oral vaccination of ruminants poses a number of challenges (i.e. degradation of the vaccine by rumen flora). It is important that the vaccine is delivered to the small intestine, where the appropriate sites for induction of mucosal immunity (Peyer's patches) are located. Thus, a delivery system is required that ensures vaccines pass safely through the rumen and efficiently target delivery to the Peyer's patches. This has been achieved in cattle with several antigens incorporated into alginate microspheres. One delivery system that appears appropriate for the BAd3 vaccine vector is alginate microspheres. These have been used to deliver live viruses since formulation does not involve the use of solvents or high temperatures. Furthermore, alginate microspheres can be formulated to the appropriate size to ensure efficient passage through the rumen.

We propose to build upon our own and other people's investigations to develop a vaccine delivery system that can provide greatly enhanced disease protection for newborn calves. The bovine adenovirus vaccine vector may provide an efficient vaccine delivery system that can be applied to numerous vaccine antigens. A marked reduction in newborn calf mortality would have significant impact on the cattle industry. An adenovirus vaccine

vector, carrying a vaccine antigen, can be easily produced in culture and should provide a relatively inexpensive vaccine. In addition, oral vaccines would avoid the deleterious effects that intramuscular/subcutaneous vaccines have on meat quality. Finally, it may be possible to combine the bovine adenovirus vaccine vectors with other non-invasive forms of vaccination (intradermal DNA vaccines; transcutaneous liposome vaccines, E. coli bacterial expression system) to provide effective disease prevention throughout the entire beef production cycle.

Objectives

The objective of this project was to develop an effective oral vaccine delivery system for the immunization of newborn calves. This was to be approached by completing the following specific Aims:

- **Assemble a microsphere production system**
- **Establish optimal production parameters for consistent microsphere size**
- **Incorporate adenovirus vaccine vector and DNA vaccines into micropsheres**
- **Monitor microsphere uptake by mucosal epithelium and Peyer's patch**
- **Confirm that microspheres can deliver vaccine antigens to immune system**
- **Monitor immune responses induced by microsphere formulated vaccines**

Project Activities, Achievements, Publications

Manuscripts

1. Kim, B., Bowersock, T., **Griebel, P.**, Kidane, A., Babiuk, LA., Sanchez, M., Attah-Poku, S., Kaushik, R.S., and G.K. Mutwiri. (2002). Mucosal immune responses following oral immunization with rotavirus antigens encapsulated in alginate microspheres. J. Controlled Release (In Press).

Presentations

1. Mutwiri, G., Bowersock, T., Kidane, A., Sanchez, M., Gerdts, V., Babiuk, L.A., and Griebel, P. (2001). Induction of mucosal immune responses following enteric immunization with antigen delivered in alginate microspheres. 6^{th} International Veterinary Immunology Symposium, Uppsala, Sweden

2. Pontarollo, R., Rankin, R., Babiuk, L.A, Godson, D., Griebel, P., Hecker, R., Krieg, A., and van Drunen Littel-van den Hurk, S. (2001). Identification of a bovine immunostimulatory CpG motif and characterization of responding cells. 6^{th} International Veterinary Immunology Symposium, Uppsala, Sweden

Research Collaborations

1. Formulation of alginate microspheres. Dr. Terry Bowerstock (Pharmacia and Upjohn).

2. Use of CpG ODN as adjuvants in cattle. Dr. Rolf Hecker (Pecura Animal Health)

3. Development of bacterial expression systems for vaccine antigens. Drs. Frank Gaertner and Steve Webb. DowAgro Sciences.

RESULTS

In this reporting period the primary objective was to determine of alginate microspheres could be used to effectively deliver an oral vaccine to young calves. For this analysis we proposed to use the bovine adenovirus 3 (BAV3) vaccine vector as a prototype commercial vaccine.

Immunogencity of the bovine adenovirus vaccine vector in the small intestine

We had previously shown that a BAV3 vaccine vector expressinga truncated form of glycoprotein D of bovine herpesvireus-1 (BAV3-tgD) could be incorporated into alginate microspheres (Aim 1). Furthermore, subcutaneous injection of alginate microspheres, containing encapsulated BAV3-tgD induced a tgD-specific immune repsonse. These observations supported the conclusion that sufficent viable BAV3-tgD vaccine vector was released from the alginate microspheres to infect host cells and induce an immune repsonse. It was necessary, however, to repeat these experiments to confirm that the BAV3-tgD vaccine vector could infect sufficient cells in the small intestine gut-associated lymphoid tissues (Peyer's patches) to induce a vaccine-specific immune response.

The intestinal "loop" model we had developed previously (Aim 2) was used to evaluate the immunogenicity of the BAV3-tgD vaccine vector. Two, 3-4 weeks old, male Holstein calves were selected for these experiments. Serum was collected prior to surgery to deterrmine if the calves might have been previously infected with either BHV-1 or BAV3. Serum antibody titres were determined using an ELISA to detect antibodies specific for either gD protein or the BAV3 virus but both calves tested negative.

To determine the immunogenicity of the BAV3-tgD vaccine vector it was necessary to first prepare a large stock of purified virus. The virus was purified using cesium chloride gradients to ensure there was no contamination by the tgD protein. Thus, any gD-specific immune response induced in the calves would be the result of tgD protein expression following infection of cells by the vaccine vector.

The intestine "loop" model was then used in the young calves to determine what dose of the BAV3-tgD vaccine vector was required to induce a detectable mucosal immune response. A total of 10 "loops" were prepared in each calf and two replicate "loops" were injected with each of the following doses of infectious BAV3-tgD vaccine vector: 0, 1 X 10^5, 1 X 10^6, 1 X 10^7, and 1 X 10^8 infectious particles. Three weeks later, the calves were euthanized and the gut-associated lymphoid tissue (Peyer's patches) were collected from each "loop" and used to assay immune responses specific for either the tgD vaccine antigen or the BAV3 vaccine vector.

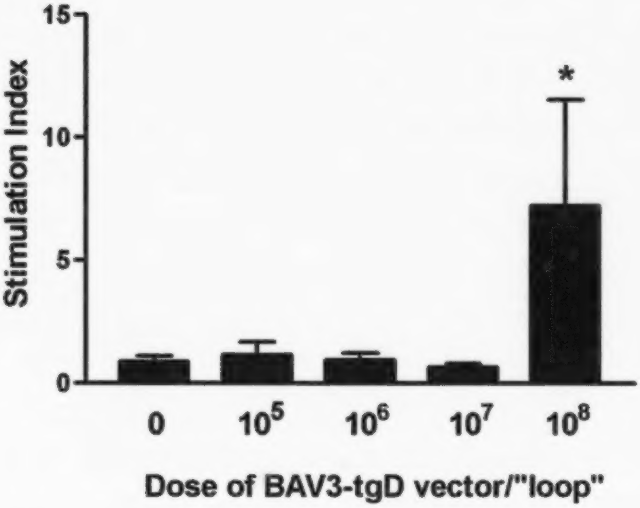

Figure 1. BAV3-specific immune response in Peyer's patches of young calves. Intestinal "loops" (n = 8) were surgically prepared in 3-4 week-old Holstein calves (n = 2) and two replicate "loops"/calf were injected with each dose of BAV3-tgD vaccine vector (n = 4/ vaccine dose). The dose of BAV3-tgD vaccine vector was defined as the number of infectious particles and ranged from no vaccine vector (0) to 1 X 10^8 infectious particles/"loop." The Peyer's patch tiisue was collected from each "loop" at three weeks after immunization and the presence of BAV3-specific immune cells (T cells) was assayed using an antigen-induced lymphocyte proliferative response (LPR) assay. A significant

LPR to BAV3 antigen was observed with cells isolated from "loops" injected with 1 X 10^8 infectious BAV3-tgD particles.

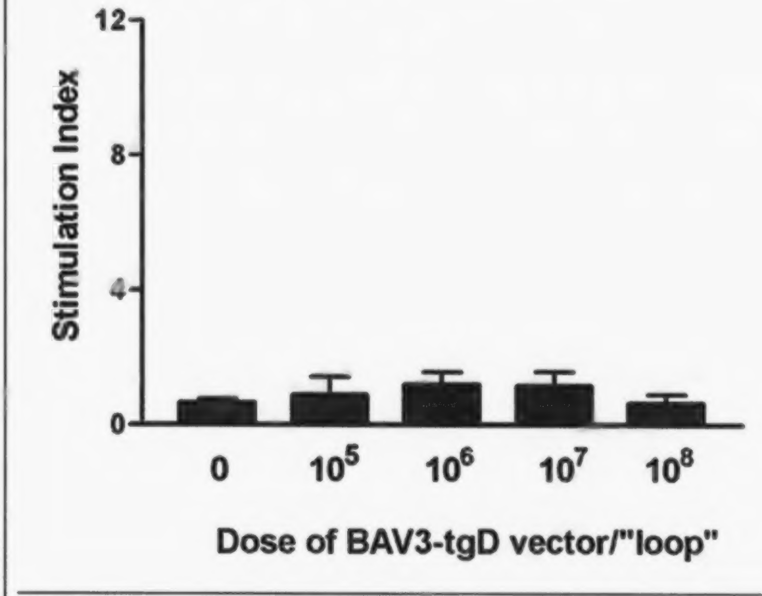

Figure 2. tgD-specific immune response in Peyer's patches of young calves. Intestinal "loops" (n = 8) were surgically prepared in 3-4 week-old Holstein calves (n = 2) and two replicate "loops"/calf were injected with each dose of BAV3-tgD vaccine vector (n = 4/ vaccine dose). The dose of BAV3-tgD vaccine vector was defined as the number of infectious particles and ranged from no vaccine vector (0) to 1 X 10^8 infectious particles/"loop." The Peyer's patch tiisue was collected from each "loop" at three weeks after immunization and the presence of tgD-specific immune cells (T cells) was assayed using an antigen-induced lymphocyte proliferative response (LPR) assay. No significant LPR to tgD antigen was observed with cells isolated from "loops" injected with 1 X 10^8 infectious BAV3-tgD particles.

The LPR assays revealed that sufficent vaccine vector was delivered to induce a specific immune repsonse to the BAV3 vector (Figure 1) but there was no detectable immune response to the encoded vaccine antigen (Figure 2). These observations confirmed

that vaccine vector was delivered to the gut-associated lymphoid tissue (Peyer's patch) but insufficient vaccine antigen was produced at this site to be immunogenic. There are several possible explanations for the failure of the BAV3-tgD vaccine vector. One possiblity is that the vaccine vector was destroyed immediately following uptake by the Peyer's patch. This would have prevented production of the encoded tgD protein. Another possibility is that viable BAV3-tgD vaccine vector enters the Peyer's patch but the cells infected by the vaccine vector do not support adenovirus replication or gene expression. Whatever the explanation for our present observations, it was apparent that the BAV3 vaccine vector did not provide an effective vaccine delivery vehicle for the induction of a mucosal immune response.

Development of an Alternative Oral Vaccine Delivery Vehicle

Rotavirus infections are an important cause of diarrhea in newborn calves and VIDO is presently licencing a new generation of rotavirus vacccines. This vaccine includes a recombinant protein (P1P5) that has been shown to provide immune protection against multiple rotavirus strains. The rotavirus vaccine being developed is for the immunization of pregnant cows. This vaccine will then pevent rotavirus infection in newborn calves through enhanced colostral transfer of maternal immunity. Therefore, we selected the P1P5 protein as a potential vaccine candidate for oral delivery to newborn calves. The first step in this selection process was to determine if the P1P5 protein could induce a mucosal immune response in the small intestine. The intestinal "loop" model in sheep was used to conduct a dose titration study with purified, soluble P1P5 protein. This study confirmed that the P1P5 protein was immunogenic and the amplitude of the mucosal immune repsonse was dependnet upon the dose of P1P5 protein injected into each intestinal "loop" (Figure 3).

The P1P5 protein is expressed in bacteria and with this expression system it is relatively easy to produce large quantities of proteins. The production of purified protein is, however, a relatively complicated process and there is considerable loss of P1P5 protein during this process. Presently, oral vaccine delivery is relatively inefficient and our previous results (Figure 3) indicated that relatively large amounts of protein (250 μg

/Peyer's patch) were required to induce a substantial mucosal immune response. Therefore, we investigated the possibility that we could use the bacterial expression system as an efficient system for both the production and delivery of a oral vaccine.

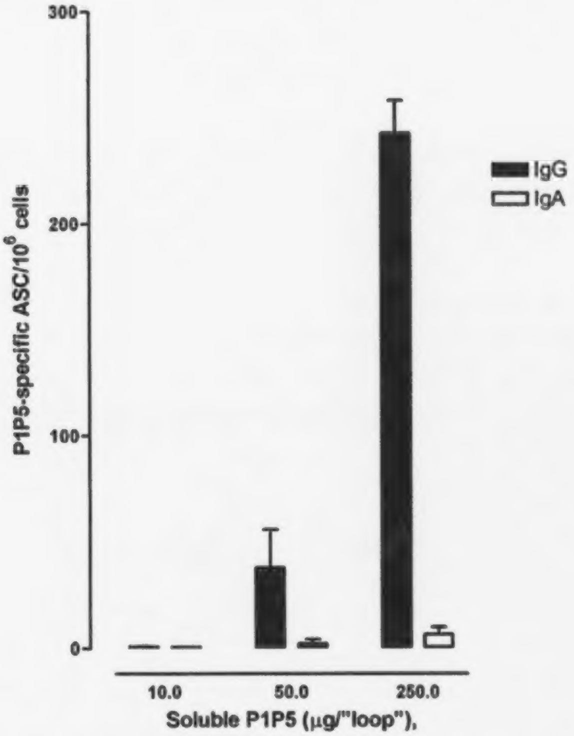

Figure 3. Soluble P1P5 protein induces a mucosal immune response. Intestinal "loops" (n = 6) were surgically prepared in 2-3 month old lambs (n = 2) and two replicate "loops"/lamb were injected with each dose of purified, soluble P1P5 rotavirus protein (n = 4/ vaccine dose). The Peyer's patch tissue was collected from each "loop" at three weeks after immunization and the presence of P1P5-specific immune cells (B cells) was assayed using an ELISPOT assay to quantify the number of IgG and IgA antibody secreting cells (ASC). There was a dose-dependent increase in the number of both IgG and IgA secreting B cells isolated from the Peyer's patches of P1P5 immunized "loops."

The P1P5 protein is produced in bacteria in an insoluble form that is referred to as an inclusion body. To test the safety and immunogenicity of the bacterial form of the oral

P1P5 rotavcirus vaccine we produced a large batch of bacteria expressing the P1P5 protein. The bacteria was then inactivated using formaldehyde fixation. The sheep intestinal "loop" model was then used to determine if this form of the rotavirus vaccine was immunogenic and what dose of formalin-fixed bacteria was required to induce a detectable mucosal immune response. The formalin-fixed bacteria induced a dose-dependent P1P5-specific mucosal immune repsonse that consisted of primarily IgG antibody secreting cells (Figure 4). Based on previous experience with the recovery of purified P1P5 protein from bacterial cultures we calculated an approximate dose of P1P5 protein for each immunized "loop." On the basis of these calculations, however, it appeared that the insoluble, bacterial encapsulated protein was not as immunogenic as purified, soluble protein.

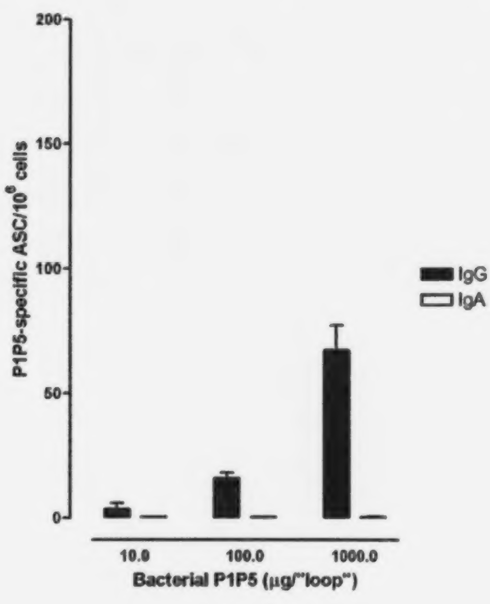

Figure 4. P1P5 protein encapsulated in formalin-fixed bacteria induces a mucosal immune response. Intestinal "loops" (n = 6) were surgically prepared in 2-3 month old lambs (n = 2) and two replicate "loops"/lamb were injected with each dose of insoluble P1P5 rotavirus protein (n = 4/ vaccine dose). The Peyer's patch tissue was collected from each "loop" at three weeks after immunization and the presence of P1P5-specific immune cells (B cells) was assayed using an ELISPOT assay to quantify the number of IgG and IgA

antibody secreting cells (ASC). There was a dose-dependent increase in the number of IgG secreting B cells isolated from the Peyer's patches of P1P5 immunized "loops."

Oral immunization of calves with P1P5 vaccine.

There are several significant barriers to overcome to achieve effective delivery of an oral vaccine. The first barrier is degradation of the vaccine in the stomach. The rumen is not fully developed in young calves and, therefore, should present less of a barrier then the abomasum. The abomasum will contain enzymes that can digest proteins and therefore destroy vaccine antigens before they reach the small intestine. We hypothesized that the formalin-fized bacteria should be more stable in the stomach and reduce degradation of the P1P5 protein. Furthermore, our previous experiments with intestinal "loops" (Figure 4) confirmed that the formalin-fixed bacteria could deliver a vaccine antigen to the gut-associated lymphoid tissue (Peyer's patches) in the small intestine. Thus, if the vaccine survived the passage through the stomach then it should be able to induce a mucosal immune rpsonse.

To determine if the formailin-fixed bacteria provided an effective oral vaccine delivery system we designed the following experiment. Serum was collected from 6-8 week old, castrated male Holstein calves to determine the level of anti-rotavirus antibody. Fifteen (15) calves were selected that had relatively low serum antibody titres (Figure 5; Day 0) and the calves were randomly allocated to three experimental groups (n = 5). Group I (Control) received oral saline and provided a control for possible exposure to rotavirus throughout the experimental period. Group II and III were orally immunized twice (Day 0 and 21) with formalin-fixed bacteria containing P1P5 inclusion bodies. The oral vaccine was prepared by pelleting the cells from 6 litres of bacterial culture and formalin fixing the cells. The cells were washed with saliine and then re-suspended in 100 ml of phosphate buffered saline to produce 10 doses (10 ml bacterin/dose) of vaccine. The oral vaccine was delivered to the rumen by passing a small diameter (OD = 0.2 cm) tube down an an esophageal feeding tube. The oral vaccine for Group III was mixed with 10 mg of a novel immune stimulatant (ODN 2007) immediately prior to delivery.

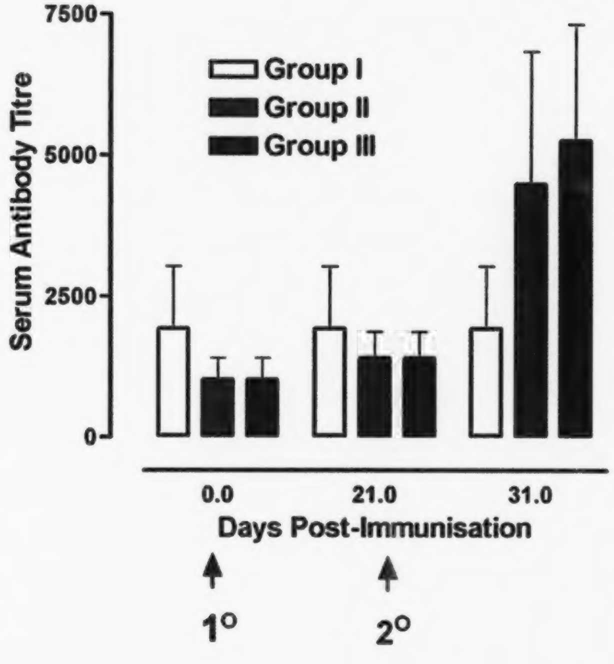

Figure 5. Oral immunization of calves with bacteria encapsulated, insoluble P1P5 protein. Calves were 6-8 weeks old at the time of the first oral immunization (1^o) and received a secondary (2^o) oral immunization 3 weeks later. Each groups of calves (n = 5) received the same vaccination for both oral immunizations. Group I received 10 ml saline, Group II received 10 ml of formalin-fixed bacterin, and Group III received 10 ml of formalin-fixed bacterin mixed with 10 mg of ODN 2007. Serum was collected from all calves on days 0, 21 and 31 following the first immunization and the level of P1P5-specific antibody was determined by ELISA.

Oral immuniation with the formalin-fixed bacterin induced a significant ($p < 0.05$) increase in P1P5-specific serum antibody titres following a secondary oral immunization (Fgiure 5). There was, however, no signifiant difference between the antibody responses of

Groiup II and III. CpG ODN has been reported to be an effective mucosal adjucvant in mice but did not have signifiant activity in young calves when orally administered in a soluble form. The induction of P1P5-specific immune responses in both Groups II and III, however, confirmed that formalin-fixed bacteria provided an effective oral vaccine delivery system.

CONCLUSIONS

1. The bovine adenovirus 3 (BAV3) vaccine vector was taken up by the Peyer's patch but failed to express sufficient vaccine antigen to induce a detectable mucosal immune response.

2. BAV3 is not an effective delivery vehicle for oral vaccines.

3. A vaccine antigen can be expressed in bacteria and formalin-fixed bacteria can effectively delivery the vaccine antigen to gut-associated lymphoid tissue (Peyer's patches) in the small intestine.

4. Oral delivery of formalin-fixed bacteria, containing an insoluble form of a vaccine antigen, results in the induction of a specific immune repsonse.

5. The induction of a specific immune response by the formalin-fixed bacterin supports the conclusion that this type of oral vaccine delivery system prevents vaccine degradation in the stomach and effectively delivers the vaccine to the immune system in the small intestine.

INFORMATION OF BENEFIT TO PRODUCERS

1. **Bovine adenovirus 3 (BAV3) is not an appropriate delivery vehicle for oral vaccines in cattle.**

2. **It is possible to express a commercial vaccine antigen (rotavirus P1P5 protein) in bacteria and use the formalin-fixed bacteria as an oral vaccine delivery system in young calves.**

3. **A bacterial expression system for vaccine antigens provides an inexpensive, large-scale production system that could produce vaccines in the quantity required for oral vaccination.**

4. **Further investigations are now in progress to identify formulations (i.e. adjuvants) that can enhance the immunogenicity of the formalin-fixed bacterin delivery system.**

5. **Oral vaccination would make it possible to immunize cattle without handling individual animals (i.e. -administer vaccine in feed supplement, mineral blocks, or top-dress feed) and could extend vaccination to new management situations (i.e. pre-immunize calves on pasture or boost cattle in feedlot pens).**

PERSONNEL

Dr. Philip Griebel	**Research Scientist**
Dr. George Mutwiri	**Research Scientist**
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Dr. Don Wilson	**Clinical Veterinarian**
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Carolyn Olsen	**Animal Health Technician**

I hereby certify that the above research has been completed.

Philip Griebel, DVM, Ph.D.

Senior Research Scientist